Endogenous Protein Inhibitors of Angiogenesis


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Endogenous protein inhibitors may serve as built-in breaks for angiogenesis. Angiogenic stimuli cause changes in endothelial cells that lead to survival, proliferation, migration, and morphogenesis. Inhibitors cause different signaling responses that usually lead to death by apoptosis. Depending upon prevailing molecular cues, angiogenesis or regression occurs.

**Pigment Epithelium-Derived Factor (PEDF)**

PEDF is a secreted glycoprotein and a member of serpin family, which actually lacks serine protease inhibitor activity and lacks an active serpin loop. It inhibits angiogenesis by promoting apoptosis of endothelial cells. It also induces neuronal differentiation. Its expression controlled by oxygen levels. The VEGF/PEDF ratio may be important for control of angiogenesis.

**Thrombospondin 1 (TSP1)**

Tsp1 is a large (180 kDa), secreted glycoprotein that has multiple functions, one of which is inhibiting angiogenesis by promoting apoptosis of endothelial cells by signaling through CD36 receptor. It is also a neurotrophic factor and can direct axon guidance. Its expression is regulated by glucose level. It also helps to maintain immune privilege by redirecting inflammation out of the eye and reducing inflammation in the eye.

Blood vessels must remodel in order to be stopped. In animals treated with VEGF inhibitors, the resident, mature blood vessels are unaffected, so quiescent vessels are not destroyed. Apotosis is dependent upon cells in G1 phase of the cell cycle. What features of growing endothelium differentiate it from quiescent endothelium that target growing endothelium for destruction by inhibitors of angiogenesis? Angiogenic stimulators increase survival kinases and antiapoptotic factors that boost endothelial cell survival and promote angiogenesis. In parallel, the same molecules produce a totally different effect: they induce a death receptor, Fas, on endothelial cell surface. However, in the absence of Fas ligand this fails to induce cell death; for that you need inhibitors of angiogenesis, such as PEDF or Tsp1, which through different signaling pathways generate Fas ligand. Fas then binds Fas ligand resulting in caspase 8-dependent apoptosis. Other inhibitors of angiogenesis, angiostatin, endostatin, and canstatin, also seem to be acting at least in part by upregulating Fas ligand and inducing Fas-dependent apoptosis in activated endothelium.

What are other points in the angiogenic cascade that can be attacked by angiogenesis inhibitors and cause endothelial cell death? Another critical molecule is nuclear factor of activated T cells or NFAT. It is involved in VEGF-induced angiogenesis. In quiescent cells, inactive NFAT is located in the cytosol. In the presence of VEGF, it translocates to the nucleus where it participates in transcriptional events. In cultured endothelial cells, NFAT is critical for VEGF-induced chemotaxis and tube formation; it is also needed for corneal angiogenesis in vivo.
How is NFAT regulated? Phosphorylation on 5 serine residues masks NFAT nuclear localization signal and maintains its cytoplasmic locale. It is bound to its regulatory phosphatase, calcineurin A, which is activated by calcium influx, which causes calmodulin activation, and in turn induction of calcineurin phosphatase. Dephosphorylation exposes NFAT nuclear localization signal so that it goes to the nucleus, binds to the consensus sites on the DNA and induces transcription. There are also opposing signals that regulate NFAT through several kinases that rephosphorylate NFAT in the cytoplasm and cause its retention, or in the nucleus, where rephosphorylation causes NFAT return to the cytoplasm through CrmA. When endothelial cells are treated with VEGF or FGF2 and cell lysates are immunoprecipitated with antiphosphoserine Ab and then immunoblotted for NFAT, they show dephosphorylation (activation) of NFAT. In control cells, NFAT is phosphorylated.

NFAT activation is disrupted by PEDF and TSP1, which cause rephosphorylation of NFAT. When cells are treated with VEGF or FGF2, staining for NFAT highlights predominantly nuclei, but when treated with both VEGF and PEDF, there are few stained nuclei. What are the kinases regulating NFAT deactivation by PEDF? PEDF has no effect on the activation of ERK kinases. JNK kinases are activated by PEDF, but only in activated endothelial cells; VEGF or FGF2 cause mild activation of JNK kinases that is greatly augmented by PEDF. Jun, a substrate for JNK kinases, is highly phosphorylated in the presence of PEDF only in stimulated and not in quiescent endothelial cells. A JNK inhibitor blocks NFAT deactivation by PEDF (dephosphorylation and nuclear localization are maintained).

Is JNK binding NFAT? JNK2, but not JNK1, binds to cytosolic NFAT. VEGF disrupts the binding of JNK1 to NFAT, but PEDF restores the binding of JNK1 to NFAT in the presence of VEGF. In the nucleus, both JNK1 and JNK2 are able to bind NFAT upon PEDF treatment. We hypothesized that JNK1 performs NFAT retention in the cytoplasm, while both JNK1 and JNK2 are responsible for the shuttling of NFAT out of the nucleus. JNK inhibitors block PEDF-induced apoptosis of endothelial cells. PEDF blocks endothelial cell migration stimulated by VEGF: JNK inhibitor disrupts this blockade. PEDF blocks FGF2-induced corneal angiogenesis, but not in the presence of a JNK inhibitor. Electrophoretic mobility shift assay demonstrates that NFAT DNA binding is reduced in PEDF-treated endothelial cells. Of NFAT targets that might be important for apoptosis, cFLIP, an endogenous inhibitor of caspase 8, was reduced in PEDF-treated cells and this reduction was JNK-dependent both at the protein and mRNA level. Chromatin immunoprecipitation assays show that NFAT binds to the cFLIP promoter in VEGF- or FGF2-treated cells: this binding is disrupted by PEDF.

In summary, VEGF or FGF2 can induce calcineurin phosphatase, which dephosphorylates NFAT, promotes its nuclear localization, and drives transcription of proangiogenic molecules such as cFLIP or Cox-2 resulting in neovascularization. However, when PEDF or TSP1 are present, there is induction of JNK kinases, which phosphorylate NFAT, causing it to relocalize to the cytoplasm thereby blocking proangiogenic transcriptional events.

Other transcription factors that are changed in remodeling endothelial cells in response to PEDF include Egr-1, NFκB, cMyb and CREB. CREB, cMyb and Er-1, like NFAT, are decreased and may form a common transcription network. CREB is a co-activator for NFAT, as is Egr-1, which can co-operate with NFAT by forming heterodimers. CMyb is one of NFAT transcriptional targets, which can also be regulated by calcium levels. NFκB, which on the contrary is decreased by PEDF, competes with NFAT for binding sites. NFκB also may drive Fas ligand expression.
The NFκB activation pathway is affected by PEDF and TSP1. IκB is an inhibitor of NFκB and its levels go down in VEGF-treated cells: this decrease is relieved by both inhibitors. IκB is regulated by phosphorylation/proteasomal degradation: phospho-IκB is increased by PEDF and by TSP1. In nuclear extracts, NFκB is increased by inducers of angiogenesis including VEGF and FGF2, but it is increased much more when PEDF or TSP1 are added. ChIP assay showed that NFκB binding to Fas ligand promoting is increased when activated endothelial cells are treated either PEDF or TSP1. VEGF or FGF2 induce NFAT activation and nuclear localization possibly in cooperation with cofactors Egr-1 and cMyb, which in turn increase levels of cFLIP, Cox2, and other targets. These signals are targeted by PEDF and TSP1 through JNK kinases. Inducers upregulate trafficking of CD95 or Fas to the endothelial cell surface, where it binds Fas ligand (also upregulated by PEDF or TSP1), causing endothelial cell apoptosis and blocking angiogenesis.

PEDF may have a special role in prostate, because PEDF KO mice have hyperplasia and increased microvascular density in the prostate. Testosterone suppresses PEDF protein levels, and PEDF is lower in metastatic prostate cancer than in non-metastatic one.

Crystal structure of PEDF indicates two areas on the surface that are likely to be involved in receptor binding consisting (44 mer and a 34 mer N-terminal peptides). The 44 mer was found to have neurodifferentiation activity, and the 34 mer was anti-angiogenic. The 34 mer, but not 44 mer (1) blocks VEGF-induced migration of endothelial cells, (2) blocks FGF2-induced angiogenesis in the corneal pocket model, (3) induces JNK activation just like full-length PEDF, (4) blocks NFAT activation, and (5) reduces binding to the cFLIP promoter.

The tet-inducible promoter system was used to express the 34 mer, the 44 mer, or a large N-terminal fragment in PC3 prostate cancer cells. The transfected cells were implanted into mice and the mice were treated with Doxycycline. The large N-terminal fragment, 34 mer, and 44 mer all decreased tumor growth, but did so in a different fashion. The N-terminal fragment and the 34 mer decreased vascular density and increased endothelial apoptosis, while the 44 mer didn’t decrease vascular density or increase endothelial apoptosis, but induced differentiation and slowed tumor growth.